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16. ABSTRACT

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GENOTOXICITY STUDIES OF BENZ[*l*]ACEANTHRYLENE

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SUMMARY

The genotoxicity of the cyclopenta-fused polycyclic aromatic hydrocarbon, benz[*l*]aceanthrylene (B[*l*]A), was evaluated in vitro using the L5178Y/TK$^{+/-}$ mouse lymphoma assay and in vivo using the mouse peripheral blood lymphocyte (PBL) culture system. The mutagenicity and sister chromatid exchange (SCE) inducing potential of B[*l*]A was then compared to that of benzo[*a*]pyrene (B[*a*]P). B[*l*]A appeared to be slightly less mutagenic than B[*a*]P at the TK locus, and each compound produced both small and large colony mutants indicating that they are clastogenic as well as mutagenic. Gross chromosome aberration analysis of treated L5178Y/TK$^{+/-}$ mouse lymphoma cells confirmed the clastogenicity of B[*l*]A in vitro. In the mouse PBL system, after administration by gavage, B[*l*]A was more cytotoxic and produced a sharper elevation in SCE frequency than B[*a*]P.

INTRODUCTION

B[*l*]A is a member of a unique class of polycyclic aromatic hydrocarbons (PAHs), the cyclopenta-fused PAHs. Various cyclopenta-fused PAHs have been identified as air pollutants, and specifically, B[*l*]A has recently been found to be a component of wood smoke emissions [18].

Cyclopenta-fused PAHs contain a fused five-membered ring, which provides a site for metabolism by the *P*-450 mixed-function oxidases [18]. Nesnow et al. [18] found that B[*l*]A was mutagenic at the HGPRT locus in V79 cells when Syrian hamster embryo cells or Aroclor-1254 induced liver

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rat S-9 were used for metabolic activation. B[*l*]A also was found to cause predominantly frameshift mutations in *Salmonella typhimurium* using an Aroclor-1254 induced rat liver S-9 fraction. Metabolism studies with this preparation indicated that cyclopenta-ring metabolism accounted for about 20% of the total metabolic products. Studies with the cyclopenta-ring oxide of B[*l*]A suggest that metabolic activation at the cyclopenta ring accounts for up to 40% of the mutagenic activity of B[*l*]A in *Salmonella typhimurium* or V79 cells. Skin tumor-initiating experiments using SENCAR mice have revealed that B[*l*]A is a potent tumor-initiating agent with approximately 4 times the activity of B[*a*]P [17].

To understand further the genotoxic potential of B[*l*]A in vitro, we compared the mutagenic activity of B[*l*]A and B[*a*]P using the L5178Y mouse lymphoma system. In addition, to evaluate the genotoxic potential of these PAHs in vivo, we examined the SCE-inducing ability of these compounds in PBLs of C57BL/6 mice following treatment by gavage.

MATERIALS AND METHODS

In vitro mutagenicity assay

The $TK^{+/-}$ L5178Y mouse lymphoma assay was used to compare the in vitro mutagenic responses of B[*l*]A and B[*a*]P. $TK^{+/-}$ 3.7.2C cells were maintained in culture and treated with either B[*l*]A or B[*a*]P according to published methods [3,4,13]. B[*a*]P (mol. wt. 252.32) was obtained from Sigma Chemical Company (St. Louis, MO), and B[*l*]A (mol. wt. 252.32) was synthesized and purified [20] by Midwest Research Institute (Kansas City, MO). Trifluorothymidine (TFT) (Sigma Chemical Co., St. Louis, MO) (1 μg/ml) was used to select for TK-deficient mutants [16]. Both B[*l*]A and B[*a*]P were dissolved in dimethyl sulfoxide and tested in medium containing 3% horse serum at a cell concentration of 0.6×10^6 cells/ml with an Aroclor 1254-induced rat liver S-9 activation system [3]. Cells were treated for 4 h and washed free of the test compound. Two days were allowed for the expression of newly induced mutants. Cells were cloned with and without TFT selection, and plates were incubated at 37°C in 5% CO_2 for 9—11 days. Colonies were counted on an Artek model 880 automatic colony counter and evaluated for their size distribution [14]. For the mutagenicity studies, the B[*l*]A and B[*a*]P treatments were performed concurrently.

In vitro chromosome aberration analysis

Separate cultures were treated for chromosome aberration studies. Following the 4-h B[*l*]A treatment, the $TK^{+/-}$ 3.7.2C cells were washed free of test compound, and 5-bromo-2′-deoxyuridine (BrdUrd) (Sigma Chemical Co., St. Louis, MO) (10 μM) was added. The harvest time was adjusted to maximize the number of first division cells. Fourteen hours after the addition of the BrdUrd, 0.1 μg/ml demecolcine (GIBCO, Grand Island, NY) was added for a 2-h incubation. Cells were treated with hypotonic KCl and fixed with acetic

acid and methanol (1 : 3). Slides were made and stained using the Fluorescence-plus-Giemsa (FPG) method [7,24].

In vivo PBL SCE assay

Animals: Male C57BL/6 mice, 8 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in the animal facility in laminar-flow rooms with 15 cycles/h of biocleaned air at 60—68% relative humidity maintained at 20—21°C. Animals were fed lab chow (Purina) and water ad libitum. The animals used in the study were 19—27 weeks of age and weighed between 30 and 35 g.

Chemical treatment: Both B[*l*]A (Midwest Research Institute, Kansas City, MO) and B[*a*]P (Imperial Industries, Cheshire, U.K.) were suspended in corn oil and administered by gavage (0.1 ml/10 g body wt). Blood was removed by cardiac puncture 23.5—25 h after treatment. B[*l*]A and B[*a*]P treatments were not done concurrently, but they were done under identical conditions*. Negative controls received only corn oil.

Blood culture: The PBLs were concentrated using a density gradient (LSM, Litton Bionetics, Kensington, MD) and washed 3 times with phosphate-buffered saline. Two 1-ml cultures were set up for each animal according to a modification [11] of the method of Erexson et al. [6]. Purified phytohemagglutinin (Burroughs Wellcome, Greenville, NC) was added at the time of culture initiation to stimulate T-cell blastogenesis, and BrdUrd (5 μM) was added 21 h later, at the time of medium change, to produce sister chromatid differentiation.

Cultures were harvested after a total culture incubation time of 50 h following a 4-h demecolcine (0.5 μg/ml) treatment. Standard hypotonic, fixation, slide preparation, and FPG staining techniques were followed [6,7, 24]. For each animal, the 2 cultures were pooled just prior to slide preparation.

Data collection and statistical analysis

Slides were coded so that the scorer had no prior knowledge of the treatment. For each concentration, 100 well-spread first division L5178Y metaphases containing 40 ± 3 chromosomes were scored for chromosome aberrations. A total of 25 second division PBL metaphases containing 40 ± 2 chromosomes were scored for SCE from each animal, and a total of 100 consecutive PBL metaphases were analysed to determine cell cycle kinetics. The replicative index (RI) [21] was calculated according to the formula:

$$RI = \text{frequency } M_1 + (2 \times \text{frequency } M_2) + (3 \times \text{frequency } M_3).$$

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*A report containing some of the B[*a*]P data as part of a World Health Organization study is in press [11].

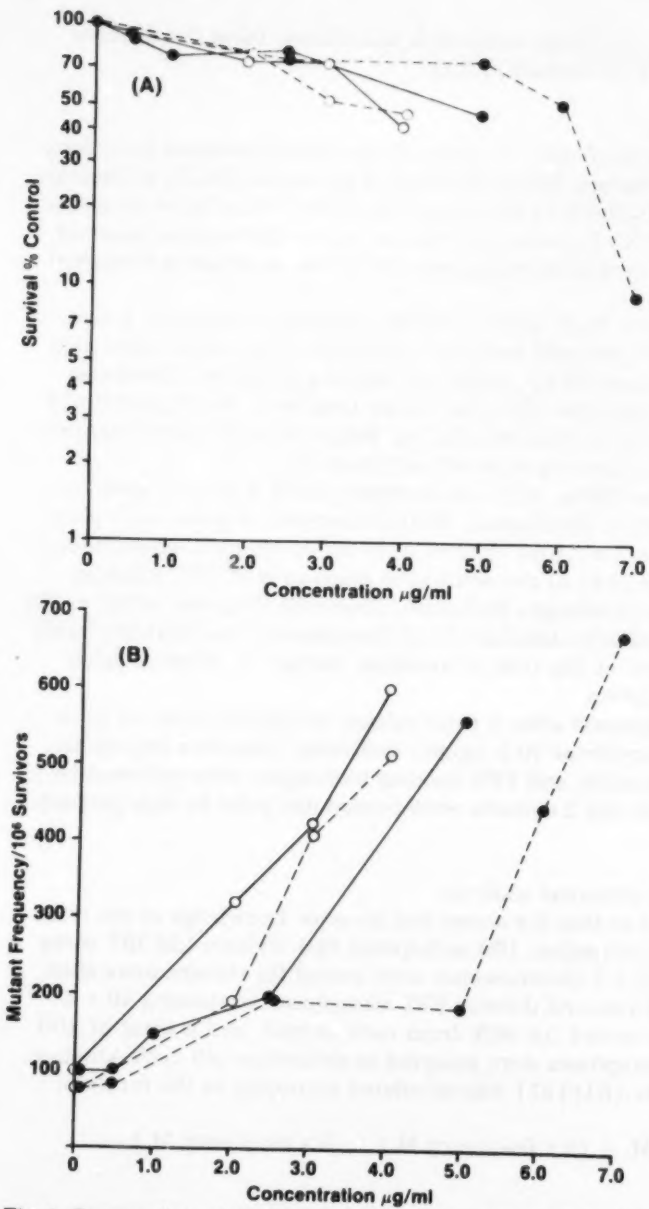

Fig. 1. Comparative cytotoxicity (A) and mutagenicity (B) of B[*l*]A (•) and B[*a*]P (○) to L5178Y/TK$^{+/-}$ mouse lymphoma cells. Survival is calculated according to the method of Clive and Spector [4] and includes both a measure of relative growth in the suspension and cloning phases of the assay. Results from the 2 separate experiments are indicated as (——) or (– – –).

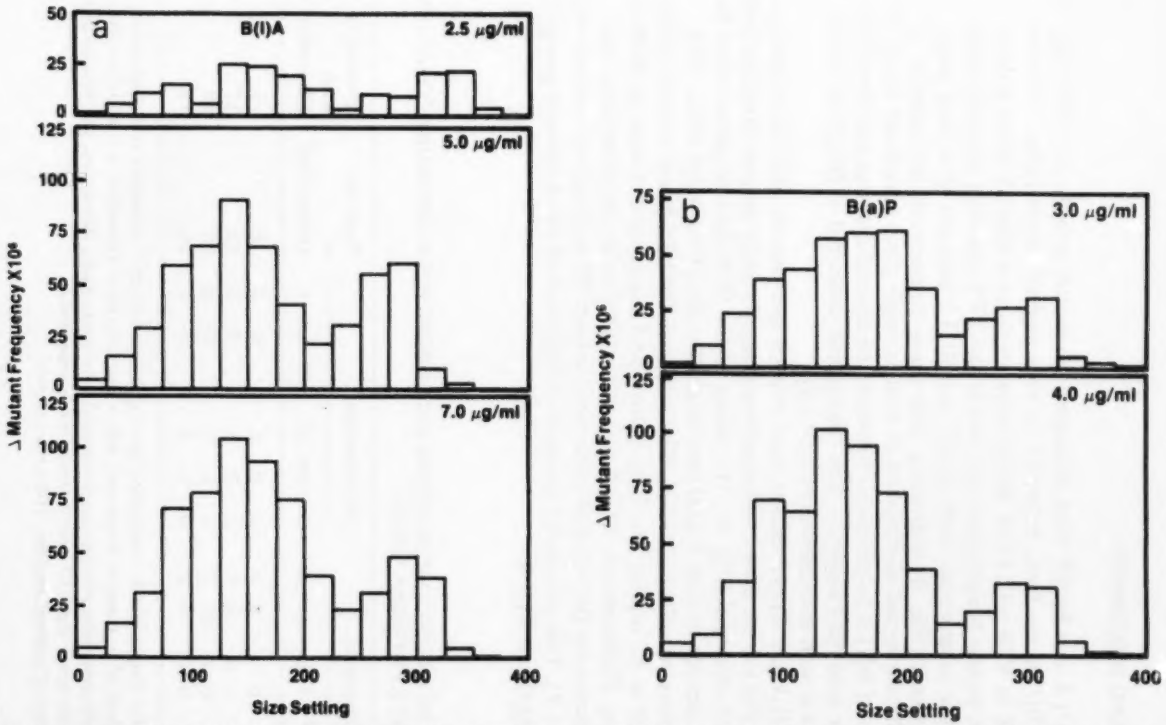

Fig. 2. Evaluation of $TK^{-/-}$-mutant colony size distribution for B[*l*]A (A) and B[*a*]P (B) treated L5178Y/$TK^{+/-}$ mouse lymphoma cells. The change in the magnitude of the mutant frequency between adjacent size settings is plotted relative to the colony counter size setting. The small-colony mutant frequency is shown as the area under the curve to the left of size setting 225. The large-colony mutant frequency is to the right of size setting 225.

The mitotic indices were calculated by determining the number of metaphases seen in 1000 consecutive PBLs. A one-way analysis of variance and Dunnett's test for comparing treatment to control groups [5] were used to determine the statistical significance of the SCE, mitotic activity, and RI data.

RESULTS AND DISCUSSION

Both B[*l*]A and B[*a*]P were mutagenic to mouse lymphoma cells (Fig. 1). Although B[*l*]A has been reported to be a slightly more potent mutagen than B[*a*]P in V79 cells [18], B[*a*]P appears to be a slightly more potent mutagen in mouse lymphoma cells. When the TFT-resistant colonies were evaluated for colony size, both B[*a*]P and B[*l*]A induced small and large colony mutants (Fig. 2) indicating that these compounds are capable of inducing chromosomal aberrations as well as single gene mutations [8,9,15]. The ability of B[*l*]A to produce chromosome aberrations was confirmed by cytogenetic analysis (Table 1). The clastogenic potential of B[*a*]P to mammalian cells is well documented [1,12,19].

Both B[*l*]A and B[*a*]P caused dose-related increases in SCE frequencies in PBLs with the lowest dose examined being significantly greater than the control for each chemical (Fig. 3). The shape of the SCE dose-response curve for B[*a*]P was similar to that found previously for B[*a*]P-exposed PBLs [10] and bone marrow cells [2,22,23]. The response for B[*l*]A was almost identical to B[*a*]P at 63 mg/kg, but B[*l*]A produced a sharper elevation in SCE at higher doses. Furthermore, B[*l*]A was more toxic at lower doses than was B[*a*]P as shown by the marked reduction in both RI and mitotic indices with dose (Table 2). This precluded analysis of SCE in 3 of the 4 animals gavaged with 252 mg/kg of B[*l*]A.

TABLE 1

ANALYSIS OF GROSS ABERRATIONS FOLLOWING B[*l*]A EXPOSURE TO L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS

Concentration (μg/ml)	Chromatid					Chromosome						Total no. of aberrations[b]	Percent cells with aberrations
	b[a]	f	tr	qr	cr	b	del	af	min	tri	t		
0	0	0	0	0	0	0	0	2	0	0	0	2	2
5.0	2	1	0	0	0	1	3	5	0	0	1	13	13
6.0	3	0	2	11	1	1	1	7	0	0	2	28	22
7.0	4	0	0	4	0	0	1	3	1	1	1	15	14

[a]b = break; f = fragment; tr = triradial; qr = quadriradial; cr = complex rearrangement; del = deletion; af = acentric fragment; min = minute; tri = tricentric; t = translocation; (NOTE: Cells also were scored for chromatid deletions, rings, dicentrics, pulverized cells, and cells with more than 10 events; none were observed).
[b]Total number of aberrations per 100 cells scored.

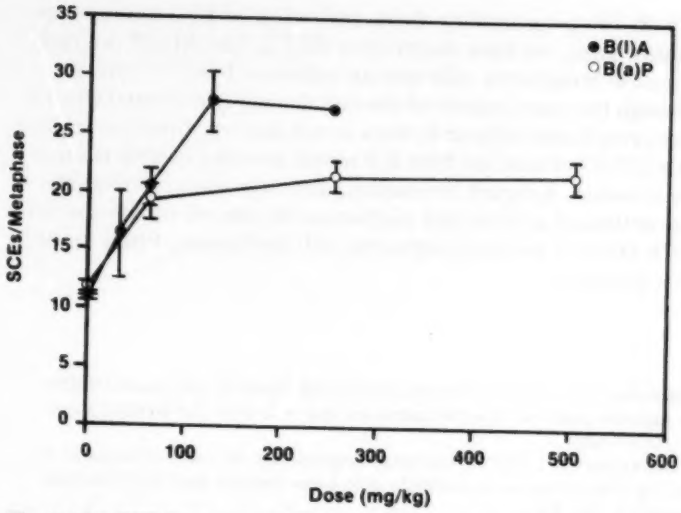

Fig. 3. The SCE frequency of PBLs of mice exposed by gavage to either B[*l*]A (•) or B[*a*]P (○). Each point represents the mean and standard deviation among 4 animals, except the 252 mg/kg dose of B[*l*]A, where only cells from 1 animal could be scored.

TABLE 2

EFFECT OF B[*l*]A AND B[*a*]P ON THE REPLICATIVE INDICES AND MITOTIC ACTIVITY OF MOUSE PBLS EXPOSED IN VIVO

Chemical	Dose (mg/kg)	Mitotic index (%)[a]	Replicative index[a]	No. of animals
B[*l*]A	0	5.1 ± 1.4	1.89 ± 0.09	4
	31.5	4.0 ± 1.1	1.84 ± 0.27	4
	63	3.5 ± 0.6	1.63 ± 0.23	4
	126	2.5 ± 0.7[b]	1.64 ± 0.17	4
	252	0.7 ± 0.3[b]	1.18[b]	4,1[c]
B[*a*]P	0	1.9 ± 1.2	1.30 ± 0.05	4
	63	1.9 ± 1.6	1.17 ± 0.07	4
	252	1.8 ± 0.9	1.22 ± 0.09	4
	504	1.6 ± 1.0	1.25 ± 0.03	4

[a]Mean ± standard deviation among animals.
[b]Significantly different from control ($P < 0.05$).
[c]Mitotic index ($n = 4$), replicative index ($n = 1$).

This study extends the genotoxicity characterization of B[*l*]A as a mammalian gene mutagen. Also, we have shown that B[*l*]A, like B[*a*]P is a clastogen in vitro in mouse lymphoma cells and an inducer of SCE in vivo in mouse PBLs. Although the exact nature of the metabolically activated species of B[*l*]A in mouse lymphoma cells or in mice is not known, previous studies [18] with Aroclor-1254 induced rat liver S-9 reveal several possible routes: cyclopenta-ring activation, k-region activation, and bay-region activation. Studies with these activated species and studies on the nature of the species which interact with DNA in mouse lymphoma cells and mouse PBLs should help to clarify this question.

REFERENCES

1 Basler, A. and Rohrborn, G. (1976) Chromosome aberrations in oocytes of NMRI mice and bone marrow cells of Chinese hamsters induced with 3,4-benzypyrene. Mutat. Res., 38, 327—332.

2 Bayer, V. and Bauknecht, T. (1977) The dose-dependence of sister chromatid exchanges induced by 3 hydrocarbons, in the in vivo bone marrow test with Chinese hamsters. Experientia, 15, 25.

3 Clive, D., Johnson, K.O., Spector, J.F.S., Batson, A.G. and Brown, M.M.M. (1979) Validation and characterization of the L5178Y/TK$^{+/-}$ mouse lymphoma mutagen assay system. Mutat. Res., 59, 61—108.

4 Clive, D. and Spector, J.F.S. (1975) Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutat. Res., 31, 17—29.

5 Dunnett, C.W. (1955) A multiple comparisons procedure for comparing several treatments with a control. J. Am. Stat. Assoc., 50, 1096—1121.

6 Erexson, G.L., Wilmer, J.L. and Kligerman, A.D. (1983) Analyses of sister chromatid exchange and cell cycle kinetics in mouse T- and B-lymphocytes from peripheral blood cultures. Mutat. Res., 109, 271—281.

7 Goto, K., Maeda, S., Kano, Y. and Sugiyama, T. (1978) Factors involved in differential Giemsa-staining of sister chromatids. Chromosoma, 66, 351—359.

8 Hozier, J., Sawyer, J., Clive, D. and Moore, M.M. (1985) Chromosome 11 aberrations in small colony L5178Y TK$^{-/-}$ mutants early in their clonal history. Mutat. Res., 147, 237—242.

9 Hozier, J., Sawyer, J., Moore, M., Howard, B. and Clive, D. (1981) Cytogenetic analysis of the L5178Y/TK+/− → TK−/− mouse lymphoma mutagenesis assay system. Mutat. Res., 84, 169—181.

10 Kligerman, A.D., Erexson, G.L. and Wilmer, J.L. (1985) Induction of sister-chromatid exchange (SCE) and cell-cycle induction in mouse peripheral blood B lymphocytes exposed to mutagenic carcinogens in vivo. Mutat. Res., 157, 181—187.

11 Kligerman, A.D., Erexson, G.L., Westbrook-Collins, B. and Allen, J.W. (1986) Analysis of sister chromatid exchange in C57BL/6 mouse peripheral blood lymphocytes following in vivo exposure to carcinogen/noncarcinogen pairs. In: Progress in Mutation Research, Evaluation of Short-Term Tests for Carcinogens — Report of the International Programme on Chemical Safety's Collaborative Study on In Vivo Assays. Editors: J. Ashby, G. Beckins, F.J. de Serres, M. Ishidate, Jr., B.H. Margolin and M.D. Shelby. Elsevier Science Publishers, Amsterdam, in press.

12 Matsuoka, A., Hayashi, M. and Ishidate, Jr., M. (1979) Chromosomal aberration tests on 29 chemicals combined with S9 mix in vitro. Mutat. Res., 66, 277—290.

13 Moore, M.M. and Clive, D. (1982) The quantitation of $TK^{-/-}$ and $HGPRT^{-}$ mutants of L5178Y/$TK^{+/-}$ mouse lymphoma cells at varying times post-treatment. Environ. Mutagen., 4, 499—519.
14 Moore, M.M., Clive, D., Howard, B.E., Batson, A.G. and Turner, N.T. (1985) In situ analysis of trifluorothymidine-resistant (TFT^{r}) mutants of L5178Y/$TK^{+/-}$ mouse lymphoma cells. Mutat. Res., 151, 147—159.
15 Moore, M.M., Clive, D., Hozier, J.C., Howard, B.E., Batson, A.G., Turner, N.T. and Sawyer, J. (1985) Analysis of trifluorothymidine-resistant (TFT^{r}) mutants of L5178Y/$TK^{+/-}$ mouse lymphoma cells. Mutat. Res., 151, 161—174.
16 Moore-Brown, M.M., Clive, D., Howard, B.E., Batson, A.G. and Johnson, K.O. (1981) The utilization of trifluorothymidine (TFT) to select for thymidine kinase-deficient ($TK^{-/-}$) mutants from L5178Y/$TK^{+/-}$ mouse lymphoma cells. Mutat. Res., 85, 363—378.
17 Nesnow, S., Gold, A.,Sangaiah, R., Triplett, L.L. and Slaga, T.J. (1984) Mouse skin-tumor-initiating activity of benz[*e*]aceanthrylene and benz[*l*]aceanthrylene in SEN-CAR mice. Cancer Letters, 22, 263—268.
18 Nesnow, S., Leavitt, S., Easterling, R., Watts, R., Toney, S.H., Claxton, L., Sangaiah, R., Toney, G.E., Wiley, J., Fraher, P. and Gold, A. (1984) Mutagenicity of cyclopenta-fused isomers of benz(a)anthracene in bacterial and rodent cells and identification of the major rat liver microsomal metabolites. Cancer Res., 44, 4993—5003.
19 Nishi, Y., Hasegawa, M.M. and Inui, N. (1980) Comparative studies on chromosome aberrations induced by polycyclic hydrocarbons in cell-mediated and microsome-mediated assay. Mutat. Res., 79, 337—344.
20 Sangaiah, R., Gold, A. and Toney, G.E. (1983) Synthesis of a series of novel poly-cyclic aromatic systems: isomers of benz(a)anthracene containing a cyclopenta-fused ring. J. Org. Chem., 48, 1632—1638.
21 Schneider, E.L. and Lewis, J. (1981) Aging and sister chromatid exchange, VIII. Effect of aging environment on sister chromatid exchange induction and cell cycle kinetics in Ehrlich ascites tumor cells. Mech. Ageing Dev., 17, 327—330.
22 Schreck, R.R. and Latt, S.A. (1980) Comparison of benzo[a]pyrene metabolism and sister chromatid exchange induction in mice. Nature (London), 288, 407—408.
23 Schreck, R.R., Paika, I.J. and Latt, S.A. (1982) Differences in murine procarcinogen activation enzymes are not accompanied by parallel differences in procarcinogen-induced sister-chromatid exchanges. Mutat. Res., 94, 143—153.
24 Wolff, S. and Perry, P. (1974) Differential Giemsa staining of sister chromatids and the study of sister chromatid exchanges without autoradiography. Chromosoma, 48, 341—353.

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